Titration of H_2O_2 in HaCaT cells

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BIOCHEMICAL SOCIETY

Aims of the project

The main objective of the study was to characterize the internalization of the second messenger H_2O_2 in the keratinocyte-like HaCaT cell line. H_2O_2 exerts a primordial role in proliferation, migration, and differentiation in all types of cells. However, the molecule is produced extracellularly and must then be transported back to the cytosol to reach its intracellular targets. The process is mediated by a family of channels called peroxiporins. As the work carried out in this project is framed in a wider project that aims to engineer lab-grown skin substitutes accurately mimicking the original tissue, it is paramount to characterize the redox processes that lead to the formation of a functional epithelium.

Methods used

Analysis of H_2O_2 transport in HaCaT cells was performed by using cells stably expressing the specific and ratiometric fluorescent HyPer7 probe in its cytosol. HyPer7 has two excitation peaks at different wavelengths (488 and 405) whose ratio of emission changes depending on its state of oxidation. Thus, when expressed intracellularly HyPer7 is a reliable indicator of the kinetics of intake after exposing the cells to a controlled challenge with H_2O_2. For conducting these measurements, HaCaT-HyPer7 cells were cultured in 6-well plates onto coverslips and then analysed with live-imaging. HyPer7 fluorescent variations were followed exposing the cells to 4 different concentrations to elaborate titrated response curves: 5µM, 10µM, 25µM, and 50µM. The process was recorded in a time span of 5 minutes, where the corresponding concentration of H_2O_2 was introduced in the culture at the 0-minute mark of the resulting plots (see Fig. 1). As control for probe oxidation, a pulse of 5mM of the reducing agent DTT was introduced at the 3-minute mark in the resulting plots (see Fig. 1). The response of the probe against time was obtained by computing the mean intensity of either fluorescence with ImageJ to obtain the ratio and plotted with GraphPad Prism. The percentage of probe activation that corresponds to the highest level of sensor activation under each condition was also calculated, using the highest concentration as reference value.

The identification of the peroxiporin isoforms responsible for the transport of H_2O_2 in HaCaT cells was performed through silencing experiments by incubation with specific siRNAs to downregulate the production of aquaporin-3 (AQP3) and 8 (AQP8), the main representants of the functional family. In this subset of experiments there was a control group (no siRNA-treated), a group treated with the siRNA for AQP3, a group treated with the siRNA for AQP8, and a final group treated with both as control for potential summatory transport efforts. All the samples were cultured as specified before and subjected to the 50µM H_2O_2 challenge while acquiring with live-imaging. The results were analysed with ImageJ and plotted with GraphPad Prism, as described above.

Results and outcomes

1. *Definition of H_2O_2 internalization curves in keratinocytes stably expressing a H_2O_2-specific ratiometric probe in the cytosol*. As shown in Fig. 1, the fold change of the HyPer7 ratio (488/405nm) increased following the concentration of H_2O_2 the cells were treated with. In every challenge applied we can discern a common behaviour. Once the H_2O_2 is introduced in the live-imaging chamber there is a sudden and rather quick spike in the HyPer7 response, which represents the increasing uptake of the molecule by HaCaT cells. Eventually, this curve hits a plateau, arriving to a maximum. As control for probe oxidation, DTT is added at the end of the course, and as expected, the ratio abruptly decreases revealing the sharp reduction of HyPer7.

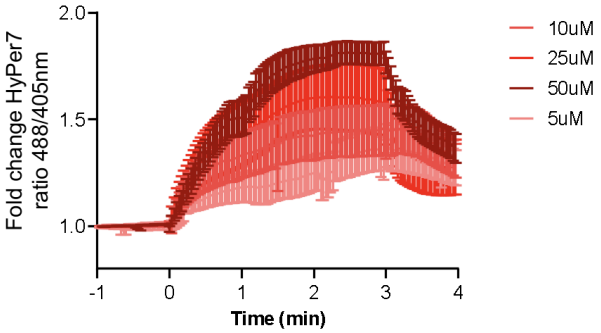


Fig. 1. Fold change ratio of HyPer7 in different concentrations of H_2O_2

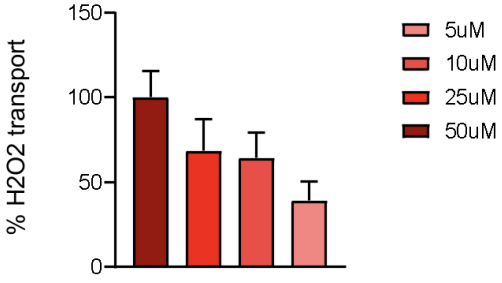


Fig 2. Percentage of H_2O_2 transport in different concentrations

The kinetical 488/405nm ratio signal of each curve can be translated into a column graph reflecting the percentage of transport of H_2O_2 into cells (Fig.2), which allows for better visualization. The highest concentration (50µM) was set as reference value. The scaled concentrations are now translated into scaled columns. Thus, we have been able to construct dose-response plots that truly reflect the capacity of transport of our cellular model. These results will be the bases for future experiments in which physiological H_2O_2 concentrations participating in fundamental cellular processes in HaCaT cells will be graduated.

2. *Determination of the channel isoform(s) controlling H_2O_2 fluxes in keratinocytes by silencing experiments*. The results of the silencing experiments shown in Fig. 3 demonstrate that both AQP3 and AQP8 isoforms participate in the transport of H_2O_2 in HaCaT cells, though with different relevance. Compared with the reference value (dark red column, labelled as 50uM in the graph), in both cases the silencing of the expression of the protein reduces the intake of H_2O_2 when applying an exogenous 50µM challenge. The siRNA for AQP8 decreased H_2O_2 transport by 30%, while the one for AQP3 had a higher impact decreasing intake by 60%. The differential capacity of transport reflects that the leading aquaporin in H_2O_2 transport in HaCaT cells would be AQP3. The double silencing that should have shown the summatory effect of both siRNAs was not informative in this subset of experiments, as in all cases only AQP3 was targeted by the silencing, while AQP8 was still expressed despite of addition of its specific siRNA (data not shown).

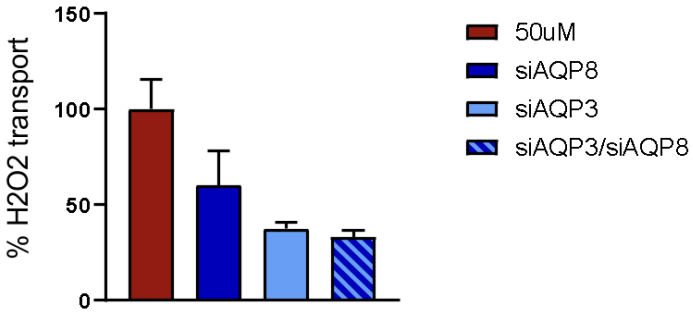


Figure 3. Percentage of H_2O_2 transport with different silencing treatments

3. *Is AQP3 redox-regulated?* Previous research of our group has shown that AQP8 can be redox-regulated resulting in a complete abrogation of H_2O_2 internalization (Medraño-Fernandez et al, 2016). The results reported were performed in HeLa a cellular model in which only AQP8 is a functional peroxiporin, and produced characteristic kinetic plots in which despite of the exogenous H_2O_2 bolus applied there was no HyPer activation. Being an oxidative modification the culprit of channel closure, the DTT that was added at the end of the course, instead of causing a decrease in the ratio, lead to a transient spike on the probe response due to AQP8 re-opening. Interestingly, during our experiments, we have observed a similar phenomenon arising in some of our samples (Fig. 4). As in HaCaT both AQP3 and AQP8 are functional H2O2 transporters this suggests that also AQP3 could be targeted by a similar gating mechanism.

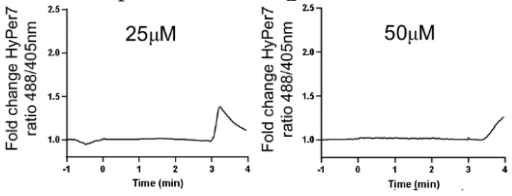


Fig. Examples of fold change ratio of HyPer7 in stressed cells

Future directions

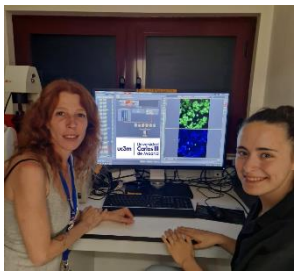
Although the results presented here are promising, more replicates should be included to reduce the error bars for statistically significant results and to firmly state the role of each peroxiporin in H_2O_2 internalization in HaCaT. However, the fact that both AQP3 and AQP8 are functional indicates that separated processes will be driven by each isoform. Investigating which signalling cascade is associated to each H_2O_2 transporter will be of undoubted interest.

Value of studentship to the student

This studentship has allowed me to transfer my knowledge obtained in the classroom and apply it to the experiments and their results. It has improved my precision on performing experiments, while increasing my resilience, patience and determination when facing failures. I have gained the ability to operate complex laboratory equipment, like a Leica Dmi8 high-velocity microscope, and I have learned to predict outcomes, analyse results, and explain anomalies using biochemical logic and scientific understanding of the topic. I have thoroughly expanded my understanding on cell culturing techniques and treatments, like silencing; detection of fluorescent probes, like HyPer7; and I have learned the importance of redox signalling, and H_2O_2 specifically, in epithelial cells. Furthermore, working as part of a research group I have been able to learn responsibility, teamwork, time management and professionalism. In this setting I have also been allowed to explore other research being carried out in the laboratory and by shadowing other team members I have been exposed to different experiments and techniques that have broaden my knowledge significantly.

Value of studentship to the research group

This project has aided the research group in their efforts to characterize the cellular and molecular processes involved skin tissue, which is paramount in order to engineer a functional epithelial tissue. It will hopefully be a step towards their end goal that is to provide adequate care to patients suffering from damaging skin conditions. This research also aligns with the Biochemical Society's strategy, by sharing the results with the scientific community promoting further research that will quicken the achievement and providing me with support and professional training as an early bioscientist.



References

1. Medraño-Fernandez et al. Stress regulates Aquaporin-8 Permeability to Impact Cell Growth and Survival. Antioxid Redox Signal. 2016 Jun 20; 24(18): 1031–1044.